Synopsis of thesis entitled

Insights into the hormonal regulation of epididymal function: a role for estrogen

submitted by

Shayu Deshpande

Department of Biochemistry

for the award of the Ph D degree of the Indian Institute of Science, Bangalore, India

---

As an organ designed for the nurture, modification and protection of sperm , the epididymis performs several functions that culminate to produce mature and fertilization-competent sperm The post-testicular modifications of sperm that occur in the epididymis are a part of the sperm maturation process, which are not inherent to the sperm but are a result of several epididymal proteins and enzymes that coat and modify the sperm membrane A highly specialized environment is maintained in the epididymis, which is formed by region specific gene and protein expression patterns within the different segments of the epididymis, i e the caput, corpus and the cauda The expression of these genes in the epididymis is tightly regulated by hormones and growth factors, the most important being androgens which are crucial for epididymal development and function In addition to androgens, recent studies have highlighted the importance of the female hormone estrogen in regulation of epididymal function A major thrust in the study of the role of estrogen in the male, stemmed from reports in the estrogen receptor alpha knockout (ERαKO) mice, which displayed disrupted fluid absorption in the excurrent ducts Testicular sperm progress from the testis to the epididymis via efferent ducts, bathed in testicular fluid that is secreted by Sertoli cells The efferent ductules and the epididymis reabsorb the testicular fluid, thus concentrating the sperm by several fold This concentration of fluid is analogous to the situation in the kidney, with which the epididymis shares its descent As in the kidney, a host of ion exchangers such as Na-K ATPase, Na-H exchangers and Cystic Fibrosis Transmembrane Regulator (CFTR) maintain ion balance in the epididymis, which governs the direction of fluid movement through the water channel proteins, aquaporins Interestingly, fluid reabsorption was severely affected in the ERαKO mice indicating a role for estrogen in regulation of this process In addition, sperm motility in the ERαKO was also drastically reduced Since sperm motility is an important parameter of sperm maturation, this was indicative of a probable dysfunction of

the epididymis Although these studies clearly highlighted the importance of estrogen in male reproduction, the precise function of estrogen in the epididymis remained elusive at the molecular level **The objectives of this study were therefore, aimed at gaining insights into the role of estrogen in the epididymis using the rat and bonnet monkey as model systems, and secondly to investigate the potential of the epididymis as a steroidogenic tissue. Since sperm maturation is a result of the region specific gene expression in the epididymis, it was also of interest to analyze the segmental gene expression within the different regions of the epididymis.**

A detailed background of the epididymis including the organization of the epididymis and its manifold functions are described in **CHAPTER 1-'General Introduction'** A brief overview of spermatogenesis and the events that lead to remodeling of spermatozoa making them capable for fertilization is presented The various hormones and growth factors that control epididymal growth and functions in general, and gene expression patterns at the molecular level in particular, are discussed at length Considering the androgen dependence of the epididymis, the details of androgen receptors and their mode of action are described Since the focus of this study was on the role of estrogen, emphasis has been placed in detailing the current literature pertaining to estrogen receptors (ER), the phenotype observed in the ERαKO and ERβKO mice and some of the estrogenic compounds and antagonists used to study the role of estrogen The chapter ends by providing the aims and scope of the present study

The details regarding the materials used and the techniques employed during the entire study are outlined in **CHAPTER 2-'Materials and Methods'** The dosage and duration of the various in-vivo treatments administered to either rats or bonnet monkeys are stated in this chapter The details of the procedures employed for isolation of RNA, Reverse Transcriptase Polymerase Chain Reaction (RTPCR), Differential Display RTPCR (DDRTPCR), Microarray, Northern Blot analysis, Western Blot analysis and Immunohistochemistry are also described The chapter also includes protocols involving Radioimmunoassay, Thin Layer Chromatography, Radioreceptor assay and protocol for analysis of cAMP production In addition, the chapter describes procedures for evaluating antibacterial activity of peptides, and includes fluorescence based membrane permeabilization and hemolysis assays Finally, details of statistical tests employed for analysis of data are presented

The results of the present study have been divided into three chapters (Chapters 3-5) The role of estrogen in the epididymis presented in **CHAPTER 3** was studied using two model systems namely, the rat and the non-human primate, bonnet monkey (*Macaca radiata*) Species-specific differences in the general organization of the epididymis and reports on species dependent differences in estrogen sensitivity led us to investigate the role of estrogen in the rodent and the monkey epididymis The outcome of these studies in the bonnet monkey also hoped to provide a better understanding of similar events in the human **A detailed analysis of the ER sub-types α and β at the mRNA and protein level showed a strong presence of the receptors along the three regions of the epididymis in both the species.** Considering that this was the first study employing bonnet monkeys to investigate the role of estrogen, a detailed localization of ERs was carried out in the three regions of the bonnet monkey epididymis Immunohistochemical localization of ERα and ERβ revealed differential localization patterns of the two receptors across the three regions of the monkey epididymis In addition, while ERα showed a strong nuclear pattern, ERβ expression was more diffuse and in addition to the intense nuclear signal, staining was observed in the cytoplasm and the surrounding stroma Since one of the most severely affected functions in the ERαKO, is fluid absorption, some of the genes involved in fluid homeostasis such as Na-K ATPase and aquaporin-1 (AQP1) were studied in the epididymides of the two species A strong expression of both the genes was observed by RTPCR analysis in the rat and monkey epididymis, albeit a differential expression was observed in the three regions of the bonnet monkey epididymis

The role of estrogen in the two species was examined by administration of an ER antagonist ICI 182780 (ICI) The expression of ERα in the rat caput and cauda regions showed a drastic reduction at both mRNA and protein level post-ICI treatment In contrast, in the monkey caput, an increase in ERα mRNA and protein expression was noted in the 30, 60, and 90 day ICI treated monkeys **However, interestingly, despite the differences in ERα expression, AQP1 mRNA level was reduced similarly in both the rat and monkey caput following ICI treatment, indicating that fluid absorption in the epididymis maybe under the sphere of estrogen control.** In order to assess whether ICI treatment actually led to an increase in fluid buildup in the excurrent ducts, monkey efferent ductules (ED) were incubated in-vitro with ICI ED were chosen due to their extreme sensitivity to lack of estrogen in comparison to the epididymis Incubation of the monkey ED with ICI in-vitro, followed by histochemical analysis, showed a 2-3 fold increase in tubule diameter due to

fluid buildup **These results together indicated a role for estrogen in regulating fluid absorption in the bonnet monkey.**

Considering the fact that an optimal functioning of the epididymis culminates to produce mature spermatozoa, defects in epididymal functioning would reflect on sperm maturation It was therefore significant to note that although there was a precipitous drop in sperm motility after ICI treatment in the bonnet monkey, sperm count was unaffected **This indicated that blockade of estrogen action by ICI was probably affecting the maturation process and not so much the sperm production process.**

As a means to identify other targets of estrogen in the epididymis, DDRTPCR was performed in the caput regions of vehicle and ICI treated bonnet monkeys **Using this technique, keratin 19 (K19), an intermediate filament was found to be down-regulated in the caput region of the 30 day ICI treated bonnet monkeys.** A reduction of K19 was also observed in the caput region of ICI treated rat Immunolocalization of K19 showed intense signal along the apico-lateral region of the epididymal epithelial cells in the normal or vehicle treated monkey, however a severe reduction in K19 expression was observed in all the ICI treated monkeys Loss of K19 in the intestine leads to an altered polarity of apical proteins In this context, the distribution of K19 in the epididymal epithelium, which is also secretory (and polarized) in nature, may be essential for anchoring several apically placed ion exchangers and proteins **Another transcript phosphotidylethanolamine N-methyl transferase (PEMT) obtained by DDRTPCR analysis, was found to be up-regulated by 90 day ICI treatment.** However, interestingly the mode of regulation of PEMT was dissimilar in the rat, where lack of androgens rather than estrogens seemed to be governing PEMT expression PEMT is involved in phosphatidylcholine biosynthesis, which is essential for maintaining fluidity of membranes and also serves as an energy source for sperm in certain species Thus, DDRTPCR analysis provided essential clues regarding a much broader role for estrogen in the epididymis These results paved the way to analyze the 'global' role of estrogen in the epididymis, by a high-throughput analysis tool such as microarray using the RNA from caput regions of wild-type and ERαKO mice Microarray analysis revealed genes involved in diverse functions to be affected by lack of ERα, e g aquaporin-4, carbonic anhydrases, secretory leukocyte protease inhibitor, phospholipase A2 V, uromodulin, etc The expression of these genes was also validated in the caput of ICI treated rat **Together, these results indicated that besides fluid**

absorption, estrogen had other gene targets in the epididymis, indicating an important role for this hormone in the epididymis.

The key role of estrogen in the epididymis prompted us to examine the potential of the epididymis for estrogen biosynthesis This premise was based on recent reports on the presence of aromatase, the enzyme involved in estrogen biosynthesis, in the epididymis Aromatase is a microsomal enzyme complex that is comprised of two proteins, the ubiquitous NADPH cytochrome P450 reductase and cytochrome P450 aromatase ($P450_{AROM}$) which possesses the heme and steroid binding pocket The enzyme complex can utilize both androstenedione and testosterone as substrates for aromatization The potential of the epididymis to function as a steroidogenic tissue was investigated, and **CHAPTER 4** presents the results of this part of the study Aromatase activity was evaluated by radioimmunoassay using a specific antibody characterized in the laboratory The cross-reactivity of the antibody against the substrates utilized for aromatase assay and other related steroids was extensively tested **Both the caput and cauda regions of the epididymis were capable of utilizing androstenedione and testosterone for aromatization.** The activity of epididymal aromatase was inhibited by the aromatase inhibitor fadrozole suggesting the presence of a functional enzyme in the epididymis **Importantly, this estradiol biosynthesis by the epididymis was devoid of any contribution from spermatozoal aromatase since all the studies employed animals of pre-pubertal age.**

Expression of $P450_{AROM}$ is regulated by luteinizing hormone (LH) and androgens in the testis, besides other growth factors The crucial role of androgens in the survival and functioning of the epididymis and the presence of LH receptors (LHR) in the epididymis, prompted us to investigate their role in regulation of epididymal $P450_{AROM}$ The removal of androgens by bilateral orchidectomy or treatment with the anti-androgen flutamide resulted in a drastic decrease in $P450_{AROM}$ expression, emphasizing the importance of testosterone in regulation of $P450_{AROM}$ expression

Diverse tissues besides the gonads have been described to express LHR, the epididymis being one of them LH is also one of the vital positive regulators of $P450_{AROM}$ expression in the Leydig cells Hence, the expression of LHR in the epididymis was analyzed in detail, and intriguingly a differential expression of LHR in the caput and cauda regions was observed in the present study, expression of LHR in the caput was surprisingly low in

comparison to the cauda region The binding of the ligand hCG (structural analog of LH), in the two regions was concurrent with the expression of LHR **Importantly, binding of hCG to cauda but not caput resulted in cAMP production, indicating a functional LHR in the cauda region.** The role of hCG in regulating $P450_{AROM}$ expression was investigated and it was observed that expression of $P450_{AROM}$ and estradiol production in the cauda was enhanced upon in-vitro treatment with hCG **Thus, both androgens and LH could regulate $P450_{AROM}$ expression in the epididymis.** In addition to aromatase, expression of other steroidogenic enzymes such as side chain cleavage enzyme, 17α-hydroxylase, 17β-hydroxy steroid dehydrogenase were observed in the epididymis and estradiol production, although low, was detected in presence of dehydroepiandrosterone and cholesterol as substrates **These results indicated the ability of the epididymis to synthesize steroids,** albeit several fold lower in comparison to the highly steroidogenic testicular Leydig cells The significance of local steroid biosynthesis as observed in the epididymis is discussed in this chapter

The ability of the epididymis to perform its diverse functions stems from its regionalized expression pattern Studies concerning specific gene expression in the epididymis are of interest in order to gain insight into the functions of the epididymis at the molecular level Using the technique of DDRTPCR, differential gene expression pattern was analyzed in the caput and cauda regions of the bonnet monkey epididymis One of the differentially expressed transcripts showed homology to a human four-disulfide-core domain containing protein called Whey Acidic Protein 10 (WAP10), which was highly expressed in the caput region **CHAPTER 5** deals with the results of this part of the study Several of the WAP proteins possess antibacterial properties due to their high basic amino acid content, by which they can penetrate the negatively charged bacterial cell wall In order to gain insight into the function of monkey WAP10 (mWAP10), peptides that had net charges of +6 (P1), +4 (P2), and '0' (P3) were designed, and their antibacterial activity was analyzed **The mWAP10 peptides employed in the present study inhibited growth of gram-negative bacterial strains of *E coli*, and significantly, the peptide P3 was ineffective in killing bacteria.** The ability of the peptides to permeabilize the bacterial cell membrane was assessed by the N-phenylnaphthylamine-fluorescence based assay Upon adding increasing concentration of the peptide P2, a dose dependent increase in fluorescence intensity was observed while the peptide P3 was unable to permeabilize bacteria, highlighting the importance of cationic charge on the peptides The peptides however, were unable to permeabilize RBCs as evidenced by the lack of hemolysis upon incubation

with the peptides This indicated an increased susceptibility of the bacterial membranes for the peptides in comparison to the mammalian membranes Proteins such as WAP10 would provide the first line of defense to the immune-privileged epididymal tract from microbial infections and thus also render protection to the male gametes sheltered within the epididymis

CHAPTER 6 provides a summary of the results obtained and a general discussion of the results in light of the current understanding in the field **In conclusion, the current investigation highlights the importance of estrogen in epididymal function across two species and identifies important targets of estrogen action in the epididymis. The study also shows the presence of a functional LHR in the epididymis and proposes the idea of the epididymis as a potential steroidogenic tissue. The differential expression of WAP10 and its antibacterial activity supports the view of the epididymis as an organ specially devised for the protection and maturation of spermatozoa**